ISSN 1346-7565

Acta Phytotax. Geobot. 67 (3): 159–174 (2016)
doi: 10.18942/apg.201607

Morphological and Molecular Studies of Natural Hybridization between *Trachelospermum asiaticum* and *Trachelospermum jasminoides* (Apocynaceae) in Japan

AZUSA UEMACHI* AND WATARU FUKUI

Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Simogamo-hangicho 1-5, Sakyo, Kyoto 606-8522, Japan.
** uemachi@kpu.ac.jp (author for correspondence)*

The species of *Trachelospermum* (Apocynaceae) are evergreen, woody lianas widely distributed in Japan. Morphologically intermediate plants between *T. asiaticum* and *T. jasminoides* have been observed. We conducted morphological and genetic investigations to clarify the possibility of hybridization events between *T. asiaticum* and *T. jasminoides*. Analysis of floral and vegetative characters of living and herbarium specimens indicated that morphologically intermediate plants frequently and widely grow in overlapping distribution areas. The results of sequencing and PCR-RFLP analyses partly confirmed natural hybridization between the two species and detected suspected hybrids among samples morphologically identical to *T. jasminoides*.

Key words: hybridization, ITS, morphology, PCR-RFLP, *Trachelospermum asiaticum, Trachelospermum jasminoides*

Trachelospermum Lem. (Apocynaceae) includes a number of evergreen, woody lianas distributed in southern and eastern Asia, with one species in southeastern North America (Murata 1989, Jussieu 1995). The species are widely used as ornamental or landscape plants around the world (Brickell 1996) and are also used in Chinese herbal medicine (Nishibe *et al*. 2002). Three species are recognized in the Japanese archipelago (Yamazaki 1993). *Trachelospermum gracilipes* Hook. f. var. *liukiuense* (Hatus.) Kitam. is restricted to the Bonin (Ogasawara) Islands and Nansei Islands southward from Yakushima (Yamazaki 1993). *Trachelospermum asiaticum* (Siebold et Zucc.) Nakai occurs westward from Iwate Prefecture, and *T. jasminoides* (Lindl.) Lem. occurs westward from the Kinki district. Therefore, the distribution areas of *T. asiaticum* and *T. jasminoides* largely overlap in western Honshu, Shikoku and Kyushu. In China, both *T. asiaticum* and *T. jasminoides* are widely distributed in central and southern regions of the country (Jussieu 1995). However, because *T. gracilipes* and *T. asiaticum* are not discriminated from each other in China (Jussieu 1995) and in Taiwan (Li & Huang 1998), the distribution area of *T. asiaticum* is unclear. *Trachelospermum jasminoides* in Japan has hirsute leaves and flowers, and has been separated as var. *pubescens* Makino from var. *jasminoides*, which occurs in China and Taiwan and has glabrous or less hirsute leaves and flowers (Hatsusima 1940, Ohwi 1965, Kitamura & Murata 1971, Murata 1989, Uemachi & Shimomura 2007, 2008). Yamazaki (1993), however, suggested that *T. jasminoides* var. *pubescens* is not distinct from var. *jasminoides*.

In areas where *Trachelospermum asiaticum* and *T. jasminoides* occur together, plants that combine their morphological characteristics have been found and the possibility of hybridization between them has been implied. A previous molecular study using nuclear internal transcribed

spacer (ITS) regions and non-coding chloroplast DNA regions revealed the phylogenetic relationships of *Trachelospermum* in Japan (Uemachi & Shimomura 2013). In that study, two individuals of *T. jasminoides* showed polymorphic patterns in their ITS sequences, indicating the presence of nucleotide sites of both *T. asiaticum* and *T. jasminoides* (Uemachi & Shimomura 2013), suggesting natural hybridization between these species. Evidence of hybridization between *T. asiaticum* and *T. jasminoides* was also reported by a random amplified polymorphic DNA (RAPD) analysis of cultivated plants of *Trachelospermum* (Uemachi & Fukui 2013).

Natural hybridization and introgression occur in many plants, and have played a significant role in their evolution (e.g., Whittemore & Schaal 1991, Rieseberg *et al*. 1996, Pellegrino *et al*. 2008). Detailed studies of hybridization and introgression are important not only from a phylogenetic standpoint, but also in terms of understanding the effects of introgression on the maintenance of species differences (Rieseberg *et al*. 1991) and the role of coadapted gene complexes in resisting the introgression of alien genes (Whittemore & Schaal 1991).

The aim of the present study was to confirm natural hybridization between *Trachelospermum asiaticum* and *T. jasminoides* in Japan by combining morphological investigations, additional sequencing of ITS regions, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the ITS regions. PCR-RFLP analyses of ITS sequences have been applied for the identification of species (e.g., Ying & Song 2003) and to detect interspecific hybrids (e.g., Moreno *et al*. 2008, Pellegrino *et al*. 2008).

Materials and Methods

Diagnostic morphological characters between species

According to previous studies (Ohwi 1965, Kitamura & Murata 1971, Murata 1989, Yamazaki 1993), *Trachelospermum asiaticum* has glabrous calyxes, pedicels, and leaves, the apex of the anther is exserted from the corolla throat, and the corolla tube is dilated at the apex (Figs. 1A & 2A). In contrast, *T. jasminoides* has hirsute calyxes and pedicels (Fig. 1B), pubescent lower surface of the leaves, the anther is included within the corolla tube, and the corolla tube is dilated above the middle (Fig. 2B). Morphological characteristics of *T. asiaticum* and *T. jasminoides* described in Yamazaki (1993) are summarized in Table 1. In the present study, the two species and intermediate forms were primarily distinguished based on these characters.

Morphological observations of herbarium specimens

We examined 97 specimens of *Trachelospermum asiaticum* and *T. jasminoides* in the Kurashiki Museum of Natural History (KURA) and 32 herbarium specimens identified as *T. jasminoides* in the Kyoto University Museum (KYO) to confirm the existence of individuals morphologically intermediate between the two species. The shape of the corolla tube, the position of the anther tip compared with the corolla throat (anther position) (Fig. 2), and the density of hairs on the calyx, pedicel, peduncle, and abaxial leaf surface were investigated. The anther position was evaluated at three levels: included, at the same height, and exserted. The density of hairs was also evaluated at three levels: glabrous, sparsely hirsute, and hirsute. In KYO, we also measured the radius of the corolla (C), corolla tube length (T), length of the narrow part of the corolla tube (N) (Fig. 3), and the calyx lobe length, with the aim of investigating the morphology of intermediate individuals in detail. The ratio of narrow part length (N) to whole length (T) of the corolla tube (N/T) was calculated.

Collection of living plant materials

Thirty eight wild individuals of *Trachelospermum* were collected from Honshu, Shikoku, and Kyushu in Japan for this study (Table 2, Fig. 4). *Trachelospermum jasminoides* from China was collected from the Ghent University Botanical Garden, Belgium (originally from the Hangzhou Botanical Garden, China) (sample No. 32),

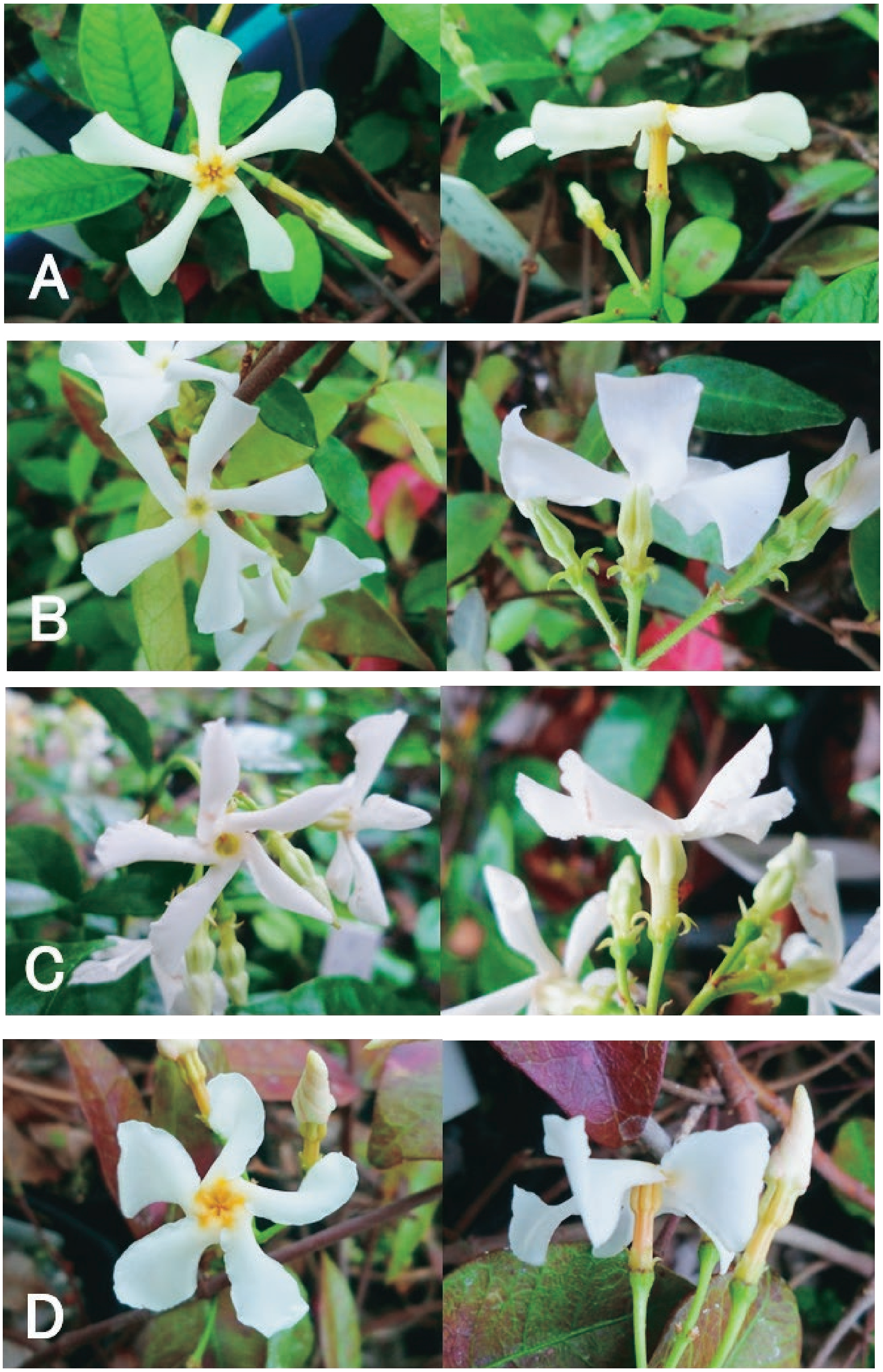

FIG. 1. Comparison of flowers of *Trachelospermum*. A: T*rachelospermum asiaticum* (sample No. 1), B: *T. jasminoides* from Japan (No. 23), C: *T. jasminoides* from China (No. 33). D: intermediate between *T. asiaticum* and *T. jasminoides* (No. 39), Sample numbers correspond to those in Table 2.

FIG. 2. The position of the anther tip compared with the corolla in *Trachelospermum*. A: exserted, *Trachelospermum asiaticum* (sample No. 5), B: included, *T. jasminoides* from Japan (No. 18). Sample numbers correspond to those in Table 2.

TABLE 1. Morphological characteristics of *Trachelospermum asiaticum* and *T. jasminoides* summarized from Yamazaki (1993).

Species	Diameter of corolla (mm)	Corolla tube length (mm)	Calyx lobe length (mm)	Shape of corolla tube	Anther position	Form of calyx	Hairs on calyx, pedicel, peduncle, and abaxial leaf surface
Trachelospermum asiaticum	15-18	7-9	3-4	Dilated at apex	Exserted	—1)	Glabrous
T. jasminoides	20-25	6-7	4-6	Dilated above the middle	Included	Reflexed	Hirsute

1) Not described.

Nakatsugawa Syubyo in Gifu Prefecture, Japan (No. 33) and Narita Nursery in Chiba Prefecture, Japan (No. 34). Two individuals of *Trachelospermum jasminoides* from Japan (Nos. 30 & 31) were collected from Nihon Groundcover in Miyazaki Prefecture in Japan. For 32 of 43 samples, voucher specimens were deposited in KYO and/or Miyazaki Prefectural Museum of Nature and History. We propagated most of the plants vegetatively at Kyoto Prefectural University to continue the observations and recorded the flowering time of individuals (Nos. 5, 14, 15, 24, 27, 30, 33 & 34).

Morphological characteristics of living plants

For 36 out of 43 samples listed in Table 2, except sample Nos. 17, 28, 29, 35, 41, 42 & 43, we obtained enough fresh flowers for morphological investigation. We measured the radius of the corolla (C), corolla tube length (T), length of the narrow part of the corolla tube (N) (Fig. 3), and the calyx lobe length. The radius of the corolla and calyx lobe length were measured at two parts of the flower and the mean value was then used. Seven to 15 flowers per plant were measured. The ratio of the length of the narrow part to the length

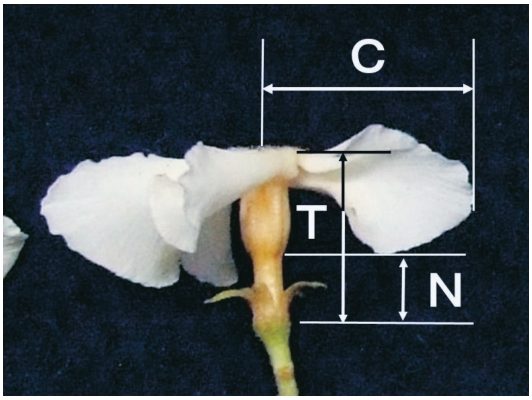

FIG. 3. Measurements of flowers. C: radius of corolla, T: corolla tube length, N: length of the narrow part of the corolla tube.

of the whole of the corolla tube (N/T) was calculated. For those values, Welch's t-tests were used to assess the significance of the differences between *Trachelospermum asiaticum* and *T. jasminoides*. We also recorded nine qualitative characters: color of the corolla lobe, corolla throat, and

TABLE 2. Source of samples used in this study.

Sample number	Species	Source	Voucher [1)]	
	Trachelospermum asiaticum			
1		Yuzamachi, Akumi-gun, Yamagata	*AU-W34-1*	
2		Daijingu, Tateyama, Chiba	*AU-W27-1*	*AU-W27-2*
3		Nikaido, Kamakura, Kanagawa	*AU-W29-1*	
4		Nishiyamacho, Atami, Shizuoka	*AU-W31-1*	
5		Kiyotaki, Maibara, Shiga	— [2)]	
6		Sawayama, Furusawacho, Hikone, Shiga	—	
7		Amatsuboyama, Serikawacho, Hikone, Shiga	—	
8		Amatsuboyama, Wadacho, Hikone, Shiga	*AU-W14-1*	
9		Mandokorocho, Higashiomi, Shiga	—	
10		Eigenjiaidanicho, Higashiomi, Shiga	—	
11		Shimogamohangicho, Kyoto Sakyo-ku, Kyoto	*AU-W53-1*	
12		Iwaneyama, Kawanishi, Hyogo	*AU-W04-1*	
13		Iwaneyama, Kawanishi, Hyogo	*AU-W13-1*	
14		Tsuruda, Nanbucho, Tottori	*AU-W11-1*	
15		Bizan, Tokushima, Tokusihma	*AU-W09-1*	
16		Yoshiki, Chikushino, Fukuoka	*AU-W44-1*	*AU-W44-2*
	T. jasminoides (from Japan)			
17		Minakuchicho, Koka, Shiga	*AU-W59-1*	
18		Kamigamomotoyama, Kyoto Kita-ku, Kyoto,	—	
19		Kamigamomotoyama, Kyoto Kita-ku, Kyoto,	—	
20		Kamigamomisonoguchicho, Kyoto Kita-ku, Kyoto	*AU-W07-1*	
21		Shimogamoizumigawacho, Kyoto Sakyo-ku, Kyoto	—	
22		Shimogamoizumigawacho, Kyoto Sakyo-ku, Kyoto	—	
23		Kajiicho, Kyoto Kamigyoku, Kyoto	*AU-W03-1*	
24		Nakaashihara, Joyo, Kyoto	*AU-W16-1*	
25		Gungehonmachi, Takatsuki, Osaka	*AU-W33-1*	
26		Gungeshinmachi, Takatsuki, Osaka	—	
27		Kitashino, Kinokawa, Wakayama	*AU-W15-1*	
28		Soja, Soja, Okayama	*AU-W23-1*	
29		Takaokacho, Miyazaki, Miyazaki	*AU-W62-1*	
30		Miyazaki prefecture, collected from a nursery	*AU-R112-1*	
31		Miyazaki prefecture, collected from a nursery	*AU-R113-1*	
	T. jasminoides (from China)			
32		Hanzhou Botanical Garden, China	*AU-S03-1*	*AU-S03-2*
33		Reputed from China, collected from a nursery	*AU-R07-1*	
34		Reputed from China, collected from a nursery	*AU-R08-1*	
	Intermediate between *T. asiaticum* and *T. jasminoides*			
35		Bodaiji, Konan, Shiga	*AU-W58-1*	
36		Kamigamomotoyama, Kyoto Kita-ku, Kyoto,	—	
37		Shimogamohangicho, Kyoto Sakyo-ku, Kyoto	*AU-W06-1*	*AU-W06-2*
38		Okazakihigashitennocho, Kyoto Sakyo-ku, Kyoto	*AU-W05-1*	*AU-W05-2*
39		Iwaneyama, Kawanishi, Hyogo	*AU-W12-1*	*AU-W12-2*
40		Yoshiki, Chikushino, Fukuoka	*AU-W35-1*	*AU-W35-2*
41		Sadowaracho, Miyazaki, Miyazaki	*K. Iwakiri, 5/13/2014**	
42		Takaokacho, Miyazaki, Miyazaki	*B-T-40105*	*B-T-40107**
43		Kaeda, Miyazaki, Miyazaki	*B-T-40170*	

1) Asterisk indicates voucher in Miyazaki Prefectural Museum of Nature and History. Other vouchers are at KYO.

2) No voucher.

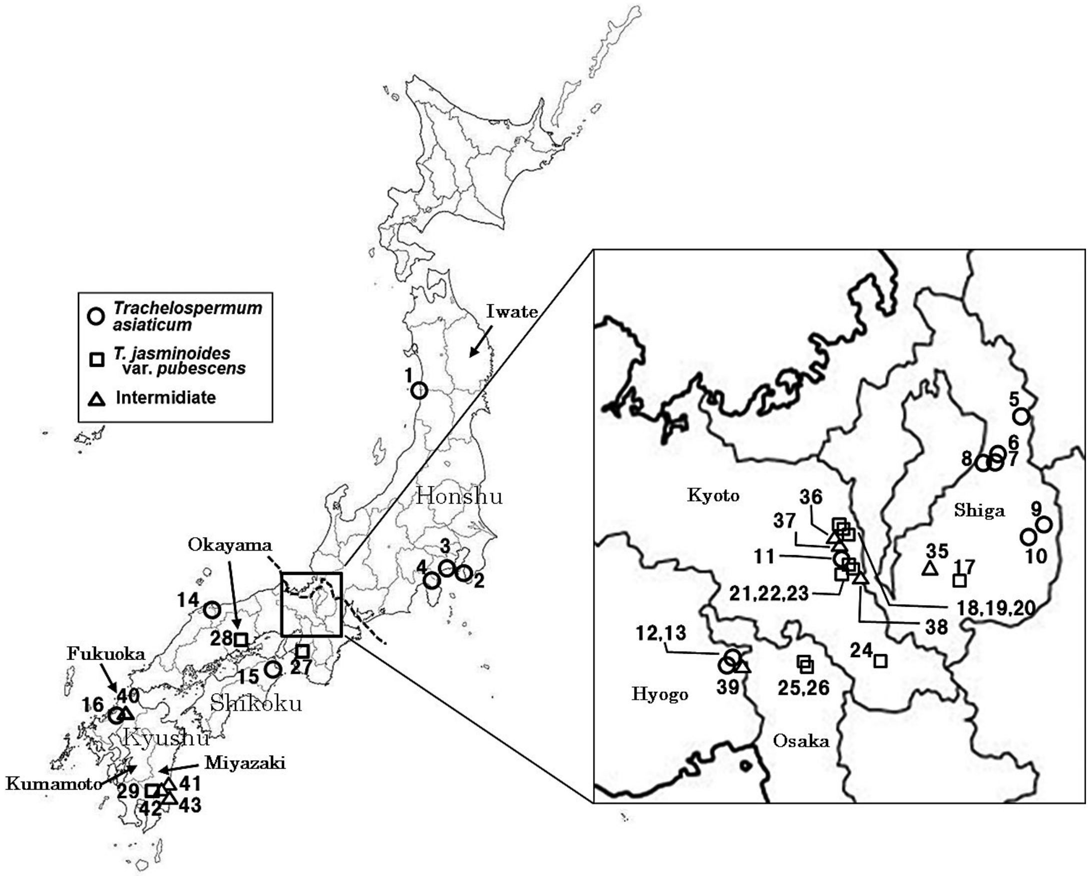

FIG. 4. Locations from which *Trachelospermum* plants were sampled in Japan.
The sample names correspond to those in Table 2. Broken line (eastern border of the Kinki district) indicates the eastern limit of distribution of *T. jasminoides*.

corolla tube, anther position (Fig. 2), form of the calyx, and density of the hairs on the three parts of the flowers (corolla throat, calyx, and pedicel and peduncle) and the abaxial surface of the leaves. The color of the corolla was determined by comparison with color cards (Practical Color Co-ordinate System). The form of the calyx was evaluated at four levels as not spreading, narrowly spreading, spreading, and reflexed. The anther position and the density of hairs were evaluated using the same method that was used for measuring herbarium specimens at KYO. For the four quantitative and nine qualitative characters, principal component analysis (PCA) was performed using Ekuseru-Toukei 2012 (Social Survey Research Information Co., Ltd., Tokyo, Japan). The quantitative characteristics were used after indexing (see Table 5).

Sequencing and PCR-RFLP analysis for the ITS regions

The ITS sequences of eight accessions were determined in this study (Table 3). DNA extraction, PCR amplification, DNA sequencing and alignment were performed as described by Uemachi and Shimomura (2013). With the sequences of 16 accessions (Uemachi & Shimomura 2013) and of two accessions of Chinese *Trachelospermum jasminoides*, we compared the ITS sequences of 26 accessions in total (Table 3).

In comparing the ITS sequences, nucleotide substitution sites without nucleotide additivity

TABLE 3. Variable sites of ITS sequences in *Trachelospermum*.

Species	Sample number [1)]	ITS (Sequence position; bp)					Accession No.	Reference
		181	224	261	334	672		
Trachelospermum asiaticum	1	A/T	A	C	T	C	AB710122	Uemachi & Shimomura 2013
	2	T	A	T	T	T	AB710123	Uemachi & Shimomura 2013
	3	T	A	T	T	T	AB610207	Uemachi & Shimomura 2013
	4	T	A	T	T	T	AB710124	Uemachi & Shimomura 2013
	8	T	A	T	T	T	AB710125	Uemachi & Shimomura 2013
	12	T	A	T	T	T	LC016851	This study
	13	A	A	C	T	C	LC016852	This study
	14	T	A	C/T	T	C/T	AB710126	Uemachi & Shimomura 2013
	15	A/T	A	C	T	C/T	AB710127	Uemachi & Shimomura 2013
	16	T	A	T	T	T	AB710128	Uemachi & Shimomura 2013
T. jasminoides from Japan	20	T	A/G	C/T	T	C/T	AB710131	Uemachi & Shimomura 2013
	23	T	G	C	C	C	LC016853	This study
	24	T	G	C	C	C	LC016854	This study
	25	T	G	C	C	C	AB710132	Uemachi & Shimomura 2013
	27	T	A/G	C	C/T	C/T	AB710133	Uemachi & Shimomura 2013
	28	T	G	C	C	C	AB610208	Uemachi & Shimomura 2013
	30	A/T	A/G	C	C/T	C	LC016855	This study
	31	T	A/G	C	C/T	C/T	LC016856	This study
T. jasminoides from China	32	T	G	C	C	C	AB710136	Uemachi & Shimomura 2013
	33	T	G	C	C	C	AB610209	Uemachi & Shimomura 2013
	34	T	G	C	C	C	AB710135	Uemachi & Shimomura 2013
	—	T	G	C	C	C	FJ980308	Chen and Han (Unpubl.)
	—	T	G	C	C	C	JF708198	Ou *et al.* (Unpubl.)
Intermediate between *T. asiaticum* and *T. jasminoides*	39	T	G	C	C	C	LC016857	This study
	40	T	G	C	C	C	AB710134	Uemachi & Shimomura 2013
	43	T	A/G	C	C/T	C	LC016858	This study

1) Sample numbers correspond to those in Table 2.

were detected between *Trachelospermum asiaticum* and *T. jasminoides* from China at sequence positions 224 and 334 (Table 3). Because sequence positions 222–225 in *T. asiaticum* were coincident with a *Spe* I restriction site (CTAG), we conducted PCR-RFLP analysis using *Spe* I in order to detect sequence differences in the morphologically intermediate plants and confirm interspecific hybridization. PCR-RFLP analysis was applied to 22 accessions (sample Nos. 1, 4, 11, 12, 14–17, 20, 23–25, 27–29, 35 & 37–42) (Table 2). PCR was performed using a primer set targeted to the ITS regions, as described previously (Uemachi & Shimomura 2013). After purification using Wizard Minipreps DNA Purification Resin (Promega, Wisconsin, USA), the PCR products were digested using *Spe* I at 37°C for more than 1 hour. The digested DNA materials were separated by gel electrophoresis in 2.0% agarose gels containing 0.4 μg/mL ethidium bromide. Gels were run for 30 min at 100 V (Mupid-2plus, Advance, Tokyo, Japan) in 1 × TAE buffer and visualized under UV light.

Results and Discussion

Morphological characteristics and flowering time

In the Kurashiki Museum of Natural History (KURA), 14 out of 97 specimens showed combined morphological characteristics of *Trachelospermum asiaticum* and *T. jasminoides*. In these morphologically intermediate specimens, the narrow part of the corolla tube was long or the apex of the anther was exserted as in *T. asiaticum*, but the leaves were hirsute as in *T. jasminoides*. All of the morphologically intermediate specimens were collected from Okayama Prefecture, Chugoku District, western Honshu (Fig. 4).

In the Kyoto University Museum (KYO), we recognized nine specimens with a combination of morphological characteristics of *Trachelosper-*

TABLE 4. Morphological characteristics of herbarium specimens (KYO) that exhibited intermediate characteristics between *Trachelospermum asiaticum* and *T. jasminoides*.

Sample number	Voucher	Location	Radius of corolla (C) (mm)	Corolla tube length (T) (mm)	Calyx lobe length (mm)	Ratio of the narrow part in the corolla tube (N/T) 1)	Anther position 2)	Hairs on flowers			Hairs on abaxial leaf surface
								Calyx	Pedicel	Peduncle	
S1	*M.Umebayashi 1503*	Mt. Hiei, Sakyo-ku, Kyoto	10.0	8.7	2.4	0.61	— 3)	Sparsely hirsute	Sparsely hirsute	Sparsely hirsute	Sparsely hirsute
S2	*M.Tagawa 3760*	Ooyamazaki, Kyoto	—	9.4	3.8	0.58	At the same height	Sparsely hirsute	Hirsute	Sparsely hirsute	Hirsute
S3	*S.Yukawa, 6/6/1996*	Kodo, Kyotanabe, Kyoto	9.8	8.8	3.7	0.60	Exserted or included	Hirsute	Sparsely hirsute	Hirsute	Sparsely hirsute
S4	*M.Tanaka 3537*	Kisaichi, Katano, Osaka	11.2	9.0	3.4	0.58	Exserted or at the same height	Hirsute	Sparsely hirsute	Hirsute	Hirsute
S5	*K.Seto 47326*	Kodachi, Yao, Osaka	10.0	9.4	4.0	0.65	Exserted	Hirsute	Sparsely hirsute	Sparsely hirsute	Sparsely hirsute
S6	*Kurosaki 17944*	Katanade, Himeji, Hyogo	13.6	8.7	4.0	0.57	Exserted or at the same height	Hirsute	Hirsute	Hirsute	Hirsute
S7	*H.Kato 940134*	Tenjinbana, Ieshima, Hyogo	8.1	7.4	3.4	0.52	Exserted	Hirsute	Hirsute	Hirsute	Hirsute
S8	*N.Fukuoka, Y.Inamasu, 5/16/1964*	Mt. Kosho-zan, Kama, Fukuoka	7.7	7.7	3.4	0.45	Exserted	Sparsely hirsute	Glabrous	Glabrous	Hirsute
S9	*K.Mayebara, 5/14/1924*	Oono, Kumamoto	12.9	7.6	5.1	0.51	Exserted	Sparsely hirsute	Sparsely hirsute	Sparsely hirsute	Hirsute

1) Ratio of the narrow part length (N) to whole length (T) in the corolla tube (see Fig. 3).
2) The position of the anther tip compared with the corolla throat (see Fig. 2).
3) Not examined.

mum asiaticum and *Trachelospermum jasminoides* among 32 specimens identified as *T. jasminoides* (Table 4). Four specimens (S5 & S7–S9) had exserted anthers similar to *T. asiaticum* and hirsute leaves similar to *T. jasminoides* (Tables 1 & 4). Although the apex of the anthers is exserted in *T. asiaticum* and included in *T. jasminoides* (Fig. 2), three specimens (S2, S4 & S6) had the apex of the anthers at the same height as the corolla throats, and one specimen (S3) bore both types of flowers. In specimen S1, the narrow part of the corolla tube was long, therefore similar to *T. asiaticum*, and the flowers and leaves were sparsely hirsute with features intermediate between the two species. These morphologically intermediate specimens were collected in Kyoto, Osaka, Hyogo, Fukuoka, and Kumamoto prefecture, in the Kinki and Kyushu districts of western Japan.

The morphological characteristics of 36 living samples are shown in Table 5. We compared the quantitative characteristics between *Trachelospermum asiaticum* and *T. jasminoides* using Welch's t-test (Table 6). From the results of this analysis, the radius of the corolla and the corolla tube length did not show a significant difference between *T. asiaticum* and *T. jasminoides*, in spite of the description of Yamazaki (1993, Table 1). The calyx lobes were significantly longer in *T. jasminoides* than *T. asiaticum*. The ratio of the narrow part of the corolla tube (N/T) in *T. asiaticum* was significantly larger than in *T. jasminoides* ($p < 0.001$), confirming the description of Yamazaki (1993; Table 1) that the corolla tube of *T. asiaticum* is dilated at the apex and that of *T. jasminoides* is dilated above the middle.

The color of the corolla was deeper in *Trachelospermum asiaticum* than in *T. jasminoides* (Table 5). The corolla lobe color was milky white or cream in *T. asiaticum*, but white in *T. jasminoides*. The color of the corolla throat was orange or near orange in *T. asiaticum*, and yellow or near yellow in *T. jasminoides*. The corolla tube color was yellow or near orange in *T. asiaticum*, and pale yellow or yellowish green in *T. jasminoides*. Yamazaki (1993) described white corolla lobes

and a yellow corolla throat for both species and did not refer to the difference between these species. Our observations confirmed a previous report on the difference in corolla color between these species based on several cultivated plants (Uemachi & Shimomura 2007).

As for hairs on the flowers, the calyx, peduncle, and pedicel were glabrous in *Trachelospermum asiaticum*, sparsely hirsute or hirsute in *T. jasminoides* from Japan, and glabrous, sparsely hirsute, or hirsute in *T. jasminoides* from China. Sample No. 32, *T. jasminoides* from China had a hirsute corolla throat, calyx, peduncle, and pedicel, thereby resembling *T. jasminoides* from Japan. In contrast, sample Nos. 33 and 34, *T. jasminoides* from China, were glabrous or sparsely hirsute and appeared to be *T. jasminoides* var. *jasminoides.*

The anther tips were exserted in *Trachelospermum asiaticum* and included in *T. jasminoides*. The calyx lobes of *T. asiaticum* were not spreading or slightly spreading while those of *T. jasminoides* were spreading, or spreading and reflexed. The abaxial leaf surface was glabrous in *T. asiaticum* and hirsute in *T. jasminoides.* These distinctions between *T. jasminoides* and *T. jasminoides* from Japan are in agreement with Yamazaki (1993; Table 1).

Morphological observations confirmed that the calyx lobe length, the ratio of the narrow part of the corolla tube (N/T), anther position, form of the calyx, and hairs on flowers and leaves can be used as diagnostic characteristics between *Trachelospermum asiaticum* and *T. jasminoides*, which is consistent with previous studies (Ohwi 1965, Yamazaki 1993, Jussieu 1995). This study and our previous report (Uemachi & Shimomura 2007) newly clarified that the corolla color can discriminate the two species. On the other hand, from the results of this study, the radius of the corolla and the corolla tube length were not diagnostic characters, which is contrary to Yamazaki (1993; Table 1).

We recognized five morphologically intermediate samples (sample Nos. 36–40) using diagnostic characters described in the materials and methods (Table 5). In sample Nos. 36, 37, 39 and 40, the anther positions were intermediate. Sample No. 38 had included anther apexes similar to *Trachelospermum jasminoides* and glabrous calyxes, pedicels, and peduncles similar to *T. asiaticum*. According to Yamazaki (1993; Table 1), the corolla tube of *T. asiaticum* is dilated at the apex and that of *T. jasminoides* is dilated above the middle. In the five samples (Nos. 36–40), the ratios of the narrow part of the corolla tube (N/T) showed intermediate values between the samples of *T. asiaticum* and *T. jasminoides* (Table 5).

We performed PCA of 13 characteristics gathered from 36 individuals (Table 5). Component 1 explained 63.5% of the total variance. Ten characteristics out of 13, except for the radius of the corolla, corolla tube length and corolla lobe color greatly contributed to this component (Table 7). Component 2 explained 12.0% of the total variance. The corolla tube length and the radius of the corolla contributed to this component (Table 7). A scatter diagram using principal components 1 and 2 (Fig. 5) showed that *Trachelospermum asiaticum* and *T. jasminoides* form two clearly separate clusters along component 1, and morphologically intermediate plants clustered separately between them.

We observed the flowering times of *Trachelospermum asiaticum* and *T. jasminoides* cultivated at Kyoto Prefectural University in 2010 (Table 8). *Trachelospermum jasminoides* from Japan (sample Nos. 24, 27 & 30) began to flower in early May, and *T. asiaticum* (Nos. 5, 14 & 15) and *T. jasminoides* from China (Nos. 33 & 34) began to flower in late May. The flowering time of *T. jasminoides* from Japan and *T. asiaticum* overlapped from late May to early June. If there is interspecific cross compatibility between these species, it seems that natural hybridization occurs in the field where both species grow together. We found morphologically intermediate plants in Shiga, Kyoto, Hyogo, Fukuoka, and Miyazaki (Table 2, Fig. 4), where both species were distributed. From these results, we presume that the morphologically intermediate plants are natural hybrids.

Considering the results of the analyses of living plants, morphologically intermediate specimens in KURA and KYO were also believed to

TABLE 5. Morphological characteristics of living samples used in this study.

Sample number [1]	Radius of corolla (C) (mm)	Corolla tube length (T) (mm)	Calyx lobe length (mm)	Ratio of the narrow part in the corolla tube (N/T) [2]	Corolla lobe color	Corolla throat color	Corolla tube color	Anther position [3]	Form of calyx	Hairs on flowers			Hairs on abaxial leaf surface
										Corolla throat	Calyx	Pedicel and peduncle	
Trachelospermum asiaticum													
1	16.1 ± 1.4[4]	10.8 ± 0.3	1.6 ± 0.4	0.66 ± 0.02	White (0)[5]	Yellow, yellowish orange, orange (3)	Yellow, yellowish green (1)	Exserted or at the same height (3)	Not spreading (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
2	10.5 ± 0.6	8.0 ± 0.3	2.0 ± 0.3	0.64 ± 0.04	Cream (2)	Yellowish orange, orange (3)	Yellow, yellowish orange, orange (3)	Exserted (4)	Not spreading (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
3	12.0 ± 1.3	9.8 ± 0.5	1.7 ± 0.2	0.66 ± 0.09	White (0)	Yellow, yellowish orange, orange (3)	Yellowish orange, orange (3)	Exserted (4)	Not spreading (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
4	10.3 ± 0.4	9.1 ± 0.3	1.3 ± 0.2	0.68 ± 0.03	White, milky white (1)	Yellowish orange (2)	Pale orange (3)	Exserted (4)	Not spreading (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
5	13.1 ± 0.6	8.6 ± 0.3	2.1 ± 0.2	0.66 ± 0.02	Milky white (1)	Orange (3)	Yellowish orange (2)	Exserted (4)	Narrowly spreading (2)	Sparsely hirsute (1)	Glabrous (0)	Glabrous (0)	Glabrous (0)
6	15.0 ± 0.5	8.6 ± 0.3	3.1 ± 0.4	0.63 ± 0.02	White (0)	Orange (3)	Pale orange, orange (3)	Exserted (4)	Narrowly spreading (2)	Glabrous or sparsely hirsute (1)	Glabrous (0)	Glabrous (0)	Glabrous (0)
7	12.6 ± 0.3	8.6 ± 0.3	2.1 ± 0.2	0.63 ± 0.02	Milky white (1)	Yellowish orange (2)	Yellowish orange, orange (3)	Exserted (4)	Narrowly spreading (2)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
8	10.8 ± 0.3	7.7 ± 0.3	2.6 ± 0.2	0.67 ± 0.03	Milky white, cream (2)	Orange (3)	Yellowish orange, orange (3)	Exserted (4)	Not spreading or narrowly spreading (1)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
9	14.0 ± 0.8	9.1 ± 0.2	2.5 ± 0.3	0.68 ± 0.02	White (0)	Yellowish orange, orange (3)	Yellowish orange, orange (3)	Exserted (4)	Not spreading (0)	Sparsely hirsute (1)	Glabrous (0)	Glabrous (0)	Glabrous (0)
10	16.1 ± 1.2	9.0 ± 0.2	3.0 ± 0.3	0.65 ± 0.03	Milky white (1)	Yellowish orange, orange (3)	Yellowish orange, orange (3)	Exserted (4)	Narrowly spreading (2)	Sparsely hirsute (1)	Glabrous (0)	Glabrous (0)	Glabrous (0)
11	11.3 ± 0.8	10.3 ± 0.4	2.3 ± 0.2	0.65 ± 0.02	Milky white, cream (2)	Yellowish orange, orange (3)	Orange, reddish orange (3)	Exserted (4)	Not spreading or narrowly spreading (1)	Sparsely hirsute (1)	Glabrous (0)	Glabrous (0)	Glabrous (0)
12	11.6 ± 0.3	9.5 ± 0.3	2.4 ± 0.2	0.65 ± 0.01	White, milky white (1)	Yellow, yellowish orange (2)	Yellow, yellowish orange (2)	Exserted (4)	Narrowly spreading (2)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
13	14.5 ± 0.8	9.2 ± 0.2	1.9 ± 0.2	0.68 ± 0.02	White, milky white (1)	Yellowish orange, orange (3)	Yellowish orange, orange (3)	Exserted (4)	Not spreading or narrowly spreading (1)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
14	10.9 ± 0.5	9.0 ± 0.4	3.5 ± 0.6	0.61 ± 0.03	Milky white (1)	Yellow, yellowish orange, orange (3)	Yellow, yellowish orange, orange (3)	Exserted (4)	Narrowly spreading (2)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
15	9.4 ± 0.7	8.9 ± 0.4	2.3 ± 0.3	0.66 ± 0.03	White (0)	Orange (3)	Yellow (1)	Exserted (4)	Narrowly spreading (2)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
16	10.5 ± 0.3	8.9 ± 0.5	1.7 ± 0.3	0.68 ± 0.04	White, milky white,	Yellow, yellowish orange (2)	Yellowish orange, orange (3)	Exserted (4)	Not spreading (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)	Glabrous (0)
Trachelospermum jasminoides from Japan													
18	13.5 ± 1.3	9.7 ± 0.3	4.0 ± 0.6	0.41 ± 0.03	Milky white, cream (2)	Pale yellow, yellow (1)	Cream, pale yellow (0)	Included (0)	Spreading (3)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
19	13.4 ± 0.7	9.8 ± 0.4	4.4 ± 0.6	0.40 ± 0.02	Milky white, cream (2)	Pale yellow, yellow (1)	Pale yellow (0)	Included (0)	Spreading (3)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
20	16.9 ± 0.9	10.6 ± 0.5	4.3 ± 0.8	0.43 ± 0.03	White (0)	Pale yellow (0)	Yellow, yellowish green (1)	Included (0)	Spreading (3)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
21	13.4 ± 0.7	8.8 ± 0.4	4.8 ± 0.4	0.46 ± 0.02	White (0)	Pale yellow, yellow (1)	Pale yellow, yellowish green (1)	Included (0)	Reflexed (4)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
22	12.5 ± 0.7	8.8 ± 0.5	4.0 ± 0.4	0.46 ± 0.04	White (0)	Pale yellow, yellowish green (1)	Yellowish green (1)	Included (0)	Spreading (3)	Hirsute (2)	Sparsely hirsute (1)	Sparsely hirsute (1)	Hirsute (2)
23	13.1 ± 0.8	8.5 ± 0.5	4.1 ± 0.5	0.41 ± 0.03	White (0)	Cream, pale yellow (0)	Yellowish green (1)	Included (0)	Reflexed (4)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
24	11.1 ± 1.4	9.3 ± 0.3	3.7 ± 0.3	0.38 ± 0.02	White (0)	Cream, pale yellow (0)	Cream, pale yellow, pale yellowish green (0)	Included (0)	Spreading (3)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
25	15.0 ± 0.7	10.4 ± 0.4	4.2 ± 0.4	0.49 ± 0.02	Milky white, cream, pale yellow (3)	Cream, pale yellow, yellowish green (1)	Yellowish green (1)	Included (0)	Reflexed (4)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)

TABLE 5. Continued.

Sample number 1)	Radius of corolla (C) (mm)	Corolla tube length (T) (mm)	Calyx lobe length (mm)	Ratio of the narrow part in the corolla tube (N/T) 2)	Corolla lobe color	Corolla throat color	Corolla tube color	Anther position 3)	Form of calyx	Hairs on flowers			Hairs on abaxial leaf surface
										Corolla throat	Calyx	Pedicel and peduncle	
Trachelospermum jasminoides from Japan (Continued)													
26	11.7 ± 0.6	7.7 ± 0.3	4.4 ± 0.4	0.43 ± 0.02	White (0)	Cream, pale yellow (0)	Cream, pale yellow, pale yellowish green (0)	Included (0)	Spreading (3)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
27	13.0 ± 0.9	9.0 ± 0.3	4.3 ± 0.4	0.34 ± 0.03	White (0)	Pale yellow, yellow (1)	Pale yellow, pale yellowish green (0)	Included (0)	Reflexed (4)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
30	13.4 ± 0.8	10.4 ± 0.6	4.0 ± 0.5	0.33 ± 0.02	White (0)	Pale yellow (0)	Cream, pale yellowish green (0)	Included (0)	Spreading (3)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
31	14.6 ± 1.0	8.6 ± 0.5	3.9 ± 0.3	0.43 ± 0.03	White (0)	Yellow, yellowish orange (2)	Yellow (1)	Included (0)	Spreading (3)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
T. jasminoides from China													
32	14.1 ± 1.5	10.2 ± 0.5	3.1 ± 0.7	0.37 ± 0.03	White, milky white (1)	Cream, pale yellow (0)	Pale yellowish green (0)	Included (0)	Reflexed (4)	Hirsute (2)	Hirsute (2)	Hirsute (2)	Hirsute (2)
33	12.9 ± 0.8	8.6 ± 0.3	4.4 ± 0.7	0.45 ± 0.03	White (0)	Pale yellow (0)	Cream, pale yellowish green (0)	Included (0)	Reflexed (4)	Glabrous (0)	Sparsely hirsute (1)	Glabrous (0)	Hirsute (2)
34	9.9 ± 0.8	7.6 ± 0.4	4.5 ± 0.7	0.46 ± 0.04	White (0)	Yellow (1)	Cream, pale yellowish green (0)	Included (0)	Reflexed (4)	Glabrous (0)	Sparsely hirsute (1)	Sparsely hirsute (1)	Hirsute (2)
Intermediate between *T. asiaticum* and *T. jasminoides*													
36	11.9 ± 0.7	8.8 ± 0.3	3.0 ± 0.3	0.53 ± 0.01	White, milky white (1)	Pale yellow, yellow (1)	Yellow, yellowish green (1)	Exserted or at the same height (3)	Reflexed (4)	Hirsute (2)	Sparsely hirsute (1)	Sparsely hirsute (1)	Hirsute (2)
37	11.5 ± 0.9	8.9 ± 0.4	2.8 ± 0.4	0.56 ± 0.03	White, milky white (1)	Yellow (1)	Yellow, yellowish green (1)	Included or at the same height (1)	Reflexed (4)	Sparsely hirsute (1)	Sparsely hirsute (1)	Sparsely hirsute (1)	Sparsely hirsute (1)
38	12.6 ± 0.7	8.4 ± 0.7	3.3 ± 0.4	0.58 ± 0.04	White, milky white (1)	Yellow, yellowish orange, orange (3)	Yellow, yellowish orange (2)	Included (0)	Narrowly spreading (2)	Hirsute (2)	Glabrous (0)	Glabrous (0)	Hirsute (2)
39	14.5 ± 1.2	8.8 ± 0.6	3.2 ± 0.4	0.59 ± 0.03	White, milky white (1)	Yellowish orange, orange (3)	Orange, reddish orange (3)	At the same height (2)	Not spreading or narrowly spreading (1)	Hirsute (2)	Sparsely hirsute (1)	Sparsely hirsute (1)	Hirsute (2)
40	11.9 ± 0.6	8.6 ± 0.4	1.9 ± 0.3	0.56 ± 0.04	White (0)	Pale yellow, yellow (1)	Pale yellow, yellowish green (1)	Included or at the same height (1)	Spreading (3)	Hirsute (2)	Sparsely hirsute (1)	Sparsely hirsute (1)	Hirsute (2)

1) The sample numbers correspond to those in Table 2.
2) Ratio of narrow part length (N) to whole length (T) in corolla tube (see Fig. 3).
3) The position of anther tip compared to corolla throat (see Fig. 2).
4) Average ± SD.
5) Converted indices used for principal component analysis (PCA). The deepest color was adopted for indexation of corolla color. Corolla lobe color: 0 = white: 1 = milky white: 2 = cream: 3 = pale yellow.
Corolla throat color & corolla tube color: 0 = pale yellow, pale yellowish green: 1 = yellow, yellowish green: 2 = yellowish orange: 3 = pale orange, orange, and reddish orange.
Anther position and form of calyx were converted into a five level scale, and existence of hair was converted into a three level scale.

TABLE 6. Comparison of morphological characteristics of *Trachelospermum asiaticum* and *T. jasminoides*.

Species	Radius of corolla (C) (mm)	Corolla tube length (T) (mm)	Calyx lobe length (mm)	Ratio of the narrow part in the corolla tube (N/T) 1)
Trachelospermum asiaticum	12.4 ± 2.1 2)	9.1 ± 0.8	2.3 ± 0.6	0.66 ± 0.02
T. jasminoides	13.2 ± 1.6	9.2 ± 1.0	4.1 ± 0.4	0.42 ± 0.05
Welch's t-test 3)	NS	NS	***	***

1) Ratio of the narrow part length (N) to whole length (T) in the corolla tube (see Fig. 3).
2) Average ± SD.
3) NS and *** represent non-significance and significance at $p < 0.001$, respectively.

TABLE 7. Principal component loading for principal component analysis in 36 individuals of *Trachelospermum asiaticum* and *T. jasminoides*.

Characteristics	Principal component	
	1	2
Radius of corolla	0.260	0.782
Corolla tube length	0.132	0.835
Calyx lobe length	0.850	-0.106
Ratio of the narrow part in the corolla tube	-0.968	0.034
Corolla lobe color	-0.258	0.248
Corolla throat color	-0.869	0.134
Corolla tube color	-0.873	0.135
Anther position	-0.949	0.051
Form of calyx	0.848	-0.206
Hair of corolla throat	0.794	0.252
Hair of calyx	0.964	0.087
Hair of pedicel and peduncle	0.931	0.125
Hair of underside of leaves	0.937	-0.075

be of hybrid origin. Therefore, adding the results of morphological observations of herbarium specimens, it turns out that the suspected hybrids occur at a significant frequency, and widely in the overlapping distribution area of both species.

ITS sequences and PCR-RFLP analysis

We compared the ITS sequences of 26 accessions and detected five nucleotide substitution sites (Table 3). Intraspecific polymorphisms and nucleotide additivities were detected in *Trachelospermum asiaticum* at sequence positions 181, 261, and 672. Neither intraspecific polymorphisms nor nucleotide additions were detected at sequence positions 224 and 334 in *T. asiaticum*. *Trachelospermum jasminoides* from China did not show polymorphisms in ITS sequences. Sequence differences without nucleotide additions were detected between *T. asiaticum* and *T. jasminoides* from China at sequence positions 224 and 334, therefore suggesting that single nucleotide polymorphisms at sequence positions 224 and 334 are useful for discriminating *T. asiaticum* from *T. jasminoides*. The sequences of *T. jasminoides* from Japan without nucleotide additions (sample Nos. 23–25 & 28) were identical to those of *T. jasminoides* from China. The remaining four samples of *T. jasminoides* from Japan (Nos. 20, 27, 30 & 31) had nucleotide additions at three of five nucleotide substitution sites respectively. Focusing on sequence positions 224 and 334, the single nucleotide substitutions between *T. asiaticum* and *T. jasminoides,* sample No. 20 showed nucleotide addition at sequence position 224, while sample Nos. 27, 30 and 31 showed additions at sequence positions 224 and 334. ITS sequences with the additions from parental sequences for hybrids have been reported by Sang *et al.* (1995), Aguilar *et al.* (1999), Rauscher *et al.* (2004), Saito *et al.* (2006), and Yano *et al.* (2010). Therefore, sample Nos. 20, 27, 30 and 31 are considered to be hybrids between *T. asiaticum* and *T. jasminoides*, although we cannot completely exclude the possibility that *T. jasminoides* from Japan has intraspecific nucleotide polymorphism at these sites.

As for the morphologically intermediate individuals, although sample No. 43 showed nucleotide additions at sequence positions 224 and 334, sequences of sample Nos. 39 and 40 were identical to those of *T. jasminoides* from China, suggesting that direct sequencing has limits in detecting hybridization between *T. asiaticum* and *T. jasminoides*.

PCR-RFLP analysis was conducted in order to detect sequence differences in the morphologically intermediate plants. PCR-RFLP results from 22 accessions are shown in Table 9. Moreover, seventeen PCR-RFLP gel patterns out of 22 accessions examined are shown in Fig. 6. *Trachelospermum asiaticum* with an *Spe* I site produced two bands at approximately 250 bp and 620 bp, whereas *T. jasminoides* without an *Spe* I site produced single band at about 870 bp. The combined band patterns are considered to indicate nucleotide additions at sequence position 224. All accessions of *T. asiaticum* identified by morphology (sample Nos. 1, 4, 11, 12, 14, 15 & 16) showed two bands, indicating the presence of the restriction site digested by *Spe* I (Table 9). This finding agreed with the ITS sequences of *T. asiaticum* (Table 3). Of eight individuals of *T. jasminoides* identified by morphology, four accessions (Nos. 23, 24, 28 & 29) showed one band, indicating no restriction site; the remaining four accessions (Nos. 17, 20, 25 & 27) showed combined band patterns. Those plants with combined band patterns might be hybrids between *T. asiaticum* and *T. jasminoides* in spite of the fact that

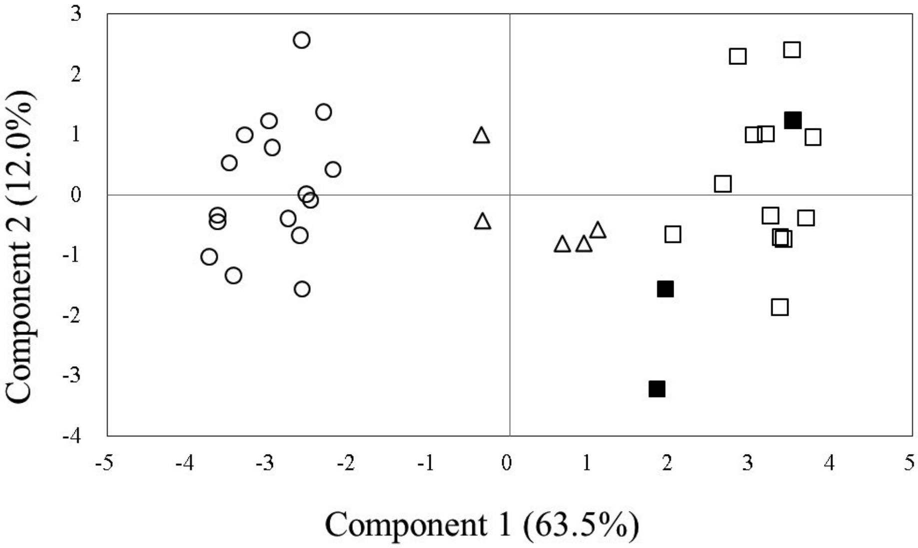

FIG. 5. Scatter diagram showing principal components 1 and 2 for 36 individuals of *Trachelospermum asiaticum* (○), *T. jasminoides* from Japan (□), *T. jasminoides* from China (■), and intermediate samples between *T. asiaticum* and *T. jasminoides* (△).

TABLE 8. Flowering times of *Trachelospermum asiaticum* and *T. jasminoides* cultivated at Kyoto Prefectural University in 2010.

Sample number[1]	Early May	Mid-May	Late May	Early June	Mid-June	Late June	Early July
Trachelospermum asiaticum							
5				◄	►		
14			◄	—	—	►	
15			◄	—	—	—	►
T. jasminoides from Japan							
24		◄	—	—	►		
27	◄	—	—	—	►		
30	◄	—	—	—	►		
T. jasminoides from China							
33				◄	—	—	— ►
34			◄	—	—	►	

1) The sample numbers correspond to those in Table 2.

they were identified as *Trachelospermum jasminoides* based on morphology.

The morphologically intermediate plants, sample Nos. 38–40 and 42 produced the expected combined band patterns. However, sample Nos. 35 and 41 showed one band at about 870 bp, and sample No. 37 showed two bands at approximately 250 bp and 620 bp. The ITS polymorphisms

resulting from hybridization are considered to be homogenized by backcrossing or concerted evolution (Sang *et al*. 1995, Aguilar *et al*. 1999, Rauscher *et al*. 2004, Yamaji *et al*. 2007a). According to Aguilar *et al*. (1999), the homogenization of polymorphisms by the effects of concerted evolution has been observed in the F_2 of artificial hybrids between two species of *Armeria*. Based on these facts and reports, incongruence of morphology and RFLP band patterns in sample Nos. 35, 37 and 41 are considered to be the result of backcrossing or concerted evolution.

Although ITS sequences of sample Nos. 25, 39 and 40 were identical to those of *Trachelospermum jasminoides* from China and did not show nucleotide additions at sequence position 224 (Table 3), the results of PCR-RFLP for these samples showed combined band patterns indicating nucleotide additivity (Table 9). PCR-RFLP analysis revealed that sample Nos. 39 and 40 with intermediate morphology had the expected nucleotide addition at sequence position 224. Yamaji *et al*. (2007b) compared the results of direct sequencing and sequencing of cloned PCR products in the ITS regions of putative hybrids in *Asarum* sect. *Asiasarum* and revealed new ribotypes not recognized in the direct sequence. Direct sequencing analysis therefore has limits in recognizing additive nucleotide sites. Those results suggest that PCR-RFLP analysis in this study detected additive nucleotide sites not detected in the direct sequence.

TABLE 9. Band patterns obtained by PCR-RFLP for the ITS regions.

Sample number[1)]	Morphologically identified species	Restriction fragment length (bp)		
		250	620	870
1	*Trachelospermum asiaticum*	+	+	−
4	*T. asiaticum*	+	+	−
11	*T. asiaticum*	+	+	−
12	*T. asiaticum*	+	+	−
14	*T. asiaticum*	+	+	−
15	*T. asiaticum*	+	+	−
16	*T. asiaticum*	+	+	−
17	*T. jasminoides*	+	+	+
20	*T. jasminoides*	+	+	+
23	*T. jasminoides*	−	−	+
24	*T. jasminoides*	−	−	+
25	*T. jasminoides*	+	+	+
27	*T. jasminoides*	+	+	+
28	*T. jasminoides*	−	−	+
29	*T. jasminoides*	−	−	+
35	Intermediate	−	−	+
37	Intermediate	+	+	−
38	Intermediate	+	+	+
39	Intermediate	+	+	+
40	Intermediate	+	+	+
41	Intermediate	−	−	+
42	Intermediate	+	+	+

1) The sample numbers correspond to those in Table 2.

As described above, we found suspected hybrids between *Trachelospermum asiaticum* and *T. jasminoides* among the progenies of cultivated *T. asiaticum* in our previous study (Uemachi & Fukui 2013). In that study, we recognized individuals with pubescent leaves in progenies of *T. asiaticum*. All of the progenies with pubescent leaves showed RAPD bands, indicating hybridization between *T. asiaticum* and *T. jasminoides*. Moreover, the RAPD bands indicated the possibility that the pollen parents of the hybrid progenies were already hybrid individuals between the two species. The findings indicated that the hybrids are able to produce fertile pollen. In the present study, we observed seed set in sample Nos. 20, 25, and 39, which we considered to be hybrids from the results of PCR-RFLP (Table 9). We sowed the seeds of sample No. 20 and observed normal germination and growth. Therefore, the hybrids are also considered to be fertile.

Furthermore, in three cpDNA regions, the sequences of *Trachelospermum jasminoides* from Japan were completely consistent with those of *T. asiaticum* and clearly differed from those of *T. jasminoides* from China (Uemachi & Shimomura 2013). *Trachelospermum jasminoides* from Japan was likely to have obtained its cpDNA from *T. asiaticum* by chloroplast capture. Congruence of cpDNA between the two species in Japan was suggested to be the cause of the frequent hybridization. Combined with these previous reports, the morphological investigation and sequencing and PCR-RFLP analysis for the ITS regions in this study indicated natural hybridization between *T. asiaticum* and *T. jasminoides* in Japan, although accurate identification of hybrids using PCR-RFLP was difficult because of the effects of backcrossing and concerted evolution in the ITS regions.

Our morphological and genetic analyses suggest that natural hybridization between sympat-

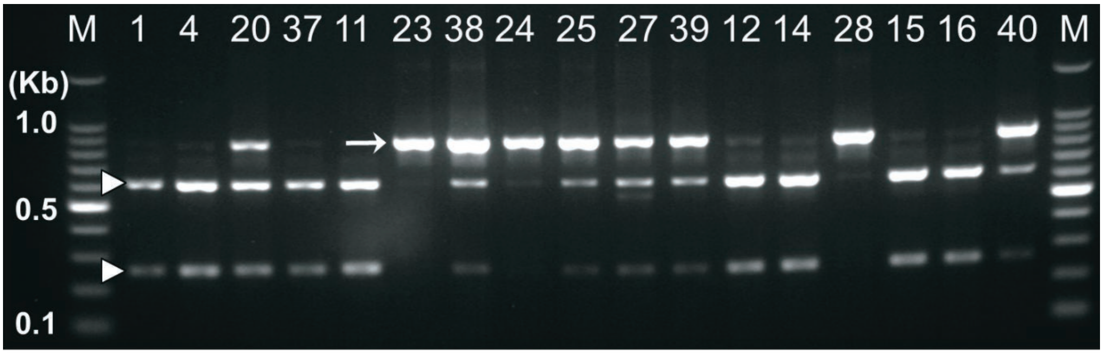

FIG. 6. RFLP profile of *Trachelospermum asiaticum* (1-16), *T. jasminoides* (20-28), and morphologically intermediate individuals (37-40). Triangles indicate fragments of about 250 bp and 620 bp, and an arrow indicates fragments of about 870 bp. Sample numbers correspond to those in Table 2. M: size marker.

ric populations of *Trachelospermum asiaticum* and *T. jasminoides* in Japan is highly probable. Considering that direct sequencing and PCR-RFLP analysis detected individuals of hybrid origin that were not detected by morphological analysis, hybridization between the two species might be more frequent than previously suspected. Due to backcrossing or to concerted evolution of ribosomal DNA, however, the genetic information based on the ITS region was insufficient for detecting hybrid individuals. To determine the actual conditions for natural hybridization in *Trachelospermum* in Japan, more extensive surveys using various nuclear markers should be conducted. Additionally, further studies using *T. asiaticum, T. jasminoides*, and related species from China, Taiwan, South Korea, and Southeast Asia are needed to clarify the extent of hybridization and introgression among these closely related taxa.

We are grateful to the late Dr. A. Kato (Kyoto Prefectural University) and Dr. M. Mino (Kyoto Prefectural University) for facilitating the molecular studies. We are also grateful to Dr. H. Nagamasu (Kyoto University Museum) and Mr. S. Kariyama (Kurashiki Museum of Natural History) for allowing us to examine specimens of *Trachelospermum*. We also thank Dr. P. Goetghebeur (Ghent University Botanical Garden), Dr. T. Shimomura (Kyoto Prefectural University), Mr. M. Saito (Miyazaki Prefectural Museum of Nature and History), Dr. T. Wada (Fukuoka Agricultural Research Center), Dr. K. Katsukawa (Hirosaki University), Dr. Wuyunbagen (Huaqiao University), Mr. A. Muranaga, Mr. J. Nozaka and Mr. T. Sumiya, for their kind help in collecting the samples.

References

Aguilar, J. F., J. A. Rossello & G. N. Feliner. 1999. Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae). Molec. Ecol. 8: 1341–1346.

Brickell, C. 1996. *Trachelospermum*. *In*: A-Z Encyclopedia of Garden Plants, p. 1018. Dorling Kindersley Limited, London.

Hatsusima, S. 1940. A revision of the Japanese *Trachelospermum*. J. Jap. Bot. 16: 20–30.

Jussieu, A. L. 1995. *Trachelospermum*. *In*: Wu, Z. Y. & P. H. Raven (eds.), Flora of China, vol. 16, pp. 166–168. Science Press, Beijing and Missouri Botanical Garden Press, St. Louis.

Kitamura, S. & G. Murata. 1971. Coloured Illustrations of Woody Plants of Japan, vol. Ⅰ, pp. 71–72. Hoikusha, Osaka (in Japanese) .

Li, H. L. & T. C. Huang. 1998. *Trachelospermum*. *In*: Flora of Taiwan Editorial Committee (eds), Flora of Taiwan, vol. 4, pp. 213–219. Flora of Taiwan Editorial Committee, Taipei.

Moreno, R., J. A. Espejo, A. Cabrera1 & J. Gill. 2008. Origin of tetraploid cultivated asparagus landraces inferred from nuclear ribosomal DNA internal transcribed spacers' polymorphisms. Ann. Appl. Biol. 153: 233–241.

Murata, G. 1989. Apocynaceae. *In*: Satake, Y., S. Watari, H. Hara & T. Tominari (eds.), Wild Flowers of Japan, Woody Plants, vol. Ⅱ, pp. 188–189. Heibonsha, Tokyo (in Japanese).

Nishibe, S., H. Y. Mei, Y. Noguchi, E. Sakai & T. Tanaka. 2002. Studies on the chinese crude drug "Luoshiteng" (3): The plant origins of Luoshiteng on the market and

their identification. Natural Medicine 56: 40–46 (in Japanese with English abstract).

Ohwi, J. 1965. Flora of Japan, revised edition. Shibundo, Tokyo (in Japanese).

Pellegrino, G., F. Bellusci & A. Musacchio. 2008. Morphological and molecular investigation of the parentage of *Ophrys* × *circlarium* (*O. lutea* × *O. tarentina*), a new hybrid orchid from Italy. Ann. Bot. Fenn. 45: 61–67.

Rauscher, J. T., J. J. Doyle & A. H. D. Brown. 2004. Multiple origins and nrDNA internal transcribed spacer homologue evolution in the *Glycine tomentella* (Leguminosae) allopolyploid complex. Genetics 166: 987–998.

Rieseberg, L. H., H. C. Choi & D. Ham. 1991. Differential cytoplasmic versus nuclear introgression in *Helianthus*. J. Hered. 82: 489–493.

Rieseberg, L. H., B. Sinervo, C. R. Linder, M. C. Ungerer & D. M. Arias. 1996. Role of gene interactions in hybrid speciation: evidence from ancient and experimental hybrids. Science 272: 741–745.

Saito, Y., M. Moller, G. Kokubugata, T. Katsuyama, W. Marubashi & T. Iwashina. 2006. Molecular evidence for repeated hybridization events involved in the origin of the genus × *Crepidiastrixeris* (Asteraceae) using RAPDs and ITS data. Bot. J. Linn. Soc. 151: 333–343.

Sang, T., D. J. Crawford & T. F. Stuessy. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequence of nuclear ribosomal DNA: Implications for biogeography and concerted evolution. Proc. Natl. Acad. U.S.A. 92: 6813–6817.

Uemachi, A. & T. Shimomura. 2007. Morphological identification of some *Trachelospermum* species using floral organs as indexes. Journal of the Japanese Society of Revegetation Technology 33: 105–110 (in Japanese with English abstract).

Uemachi, A. & T. Shimomura. 2008. A study for easy identification of species of the genus *Trachelospermum* using native specimens from the middle Kinki district and plants commercially distributed in Japan. Journal of the Japanese Society of Revegetation Technology 34: 115–120 (in Japanese with English abstract).

Uemachi, A. & T. Shimomura. 2013. Molecular phylogeny of *Trachelospermum* (Apocynaceae) in Japan based on cpDNA and nrDNA nucleotide sequences. Acta Phytotax. Geobot. 64: 1–13.

Uemachi, A. & W. Fukui. 2013. Identification of *Trachelospermum* plants and detection of hybrids using RAPD analysis. Journal of the Japanese Society of Revegetation Technology 39: 9–14 (in Japanese with English abstract).

Whittemore, A. T. & B. A. Schaal. 1991. Interspecific gene flow in sympatric oaks. Proc. Natl. Acad. Sci. U.S.A. 88: 2540–2544.

Yamaji H., T. Fukuda, J. Yokoyama, J. Pak, C. Zhou, C. Yang, T. Morita & M. Maki. 2007a. Hybridization and chloroplast captures in *Asarum* sect. *Asiasarum* (Aristolochiaceae) documented by chloroplast DNA sequences. Acta Phytotax. Geobot. 58: 87–96.

Yamaji, H., T. Fukuda, J. Yokoyama, J. Pak, C. Zhou, C. Yang, K. Kondo, T. Morita, S. Takeda, H. Sasaki & M. Maki. 2007b. Reticulate evolution and phylogeography in *Asarum* sect. *Asiasarum* (Aristolochiaceae) documented in internal transcribed spacer sequences (ITS) of nuclear ribosomal DNA, Molec. Phylogen. Evol. 44: 863–884.

Yamazaki, T. 1993. *Trachelospermum*. *In*: Iwatsuki, K., T. Yamazaki, D. E. Boufford and H. Ohba (eds.), Flora of Japan 3a, pp. 166–167. Kodansha, Tokyo.

Yano, O., H. Ikeda & T. Hoshino. 2010. Molecular and cytological studies of an interspecific hybrid in *Schoenoplectus* (Cyperaceae). Acta Phytotax. Geobot. 60: 141–149.

Ying, B. & G. Song. 2003. Identification of *Oryza* species with the CD genome based on RFLP analysis of nuclear ribosomal ITS sequences. Acta Bot. Sin. 45: 762–765.

Received September 14, 2014; accepted February 25, 2016